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ROLE OF A NOVEL STARCH BRANCHING ENZYME IN BIOSYNTHESIS OF BARLEY A-TYPE STARCH GRANULES

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Final Report

Role of a Novel Starch Branching Enzyme in the Biosynthesis of Barley A-type Starch Granules.

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Abstract

Starches extracted from wheat, barley and rye contain two size fractions of starch granules, large and small. We have in our previous work shown that a 150 kD starch branching enzyme I (SBEIc) is preferentially incorporated into large starch granules, whereas the 87 kD SBEI is found in both small and large granules. The previous work has indicated that SBEIc has a role in determining the size distribution of starch granules within the Triticeae tribe of cereals. In this project we have characterized the barley genomic region encoding SBEIc. An unusual arrangement of SBEI and SBEIc coding sequences were found and two novel SBEI-like genes were also predicted from a 100 kb DNA sequence. Proteins corresponding in size to peptides encoded by the *Sbe1*-like genes were, like SBEIc and SBEI, bound to starch granules. Expression of one of the small *Sbe1*-like genes was predicted to be coordinated with expression of *Sbe1c* through a splicing mechanism. Thus, the ratio of small SBEI-like peptides and SBEIc incorporated into starch granules appeared to be coordinated and may all control the biogenesis of small and large granules. Two other proteins encoded by genes associated with chromosome 2 QTL for starch granule size were also found to be attached to starch granules. However, these proteins did not differ in abundance between small and large granules. About 152 different barley lines have been grown in a greenhouse and used for study of starch granules. The analysis identified three lines with low B granule numbers and high A granule volume, which are starch granule qualities desired for malting purposes. Further studies will be done on these lines to determine if SBEIc or any of the four starch granule-associated proteins identified in this study is altered in its expression/activity in these lines.

Executive summary

The following objectives were set for this study:

1. Construction of a barley cDNA library from developing kernel.
   Isolation of a full-length cDNA encoding SBEIc homologue in barley.
2. Characterization of isolated cDNA by DNA sequencing.
3. Screening of 1000 barley lines from diverse regions of the world for granule size distribution and correlate these values with *Sbe1c* expression levels.

Instead of constructing a cDNA library as set out in our original proposal, the genomic region encoding *Sbe1c* in barley was isolated instead and analyzed by DNA sequencing. Using the DNA sequence information generated, the *Sbe1c* cDNA was obtained by RT-PCR. This change in the plan allowed us to compare the sequence of the *Sbe1c* transcript to the genomic region encoding *Sbe1c*. The DNA sequence of a 100 kb region of barley genome revealed that the well-characterized 87 kD SBEI gene was closely linked to two small SBEI-like genes, one of which showed sequence homology to the first part of the *Sbe1c* coding region. Surprisingly, a continuous DNA region encoding *Sbe1c* was not found. Rather, the analysis predicted that *Sbe1c* mRNA is formed by joining one of the *Sbe1-like* transcripts with the latter part of the *Sbe1* mRNA through a trans-splicing event or by alternative splicing. In addition to mRNA encoding the 150 kD SBEIc and the 87 kD SBEI, the two small *Sbe1*-like transcripts were found to be expressed during accumulation of starch in developing kernels. Polypeptides corresponding in size to the predicted small SBEI-like proteins and recognized by SBEI antibodies were also found in starch granules, thus suggesting a role for these polypeptides in starch biosynthesis or

degradation. In contrast to SBEIc, the two novel proteins did not differ in abundance between small and large starch granules. Since expression of the small *SbeI*-like transcripts and *SbeIc* appears to be tightly linked through an unusual splicing mechanism, the ratio of encoded proteins accumulated in granules will also be under tight control. This may play a role in the biogenesis of large and small granules, which are initiated at different time points during grain filling. Finding mutants in genes encoding the small SBEI-like proteins or SBEIc will be important for understanding the role of these proteins.

Two genes (gene A and B) involved in glucan biosynthesis were located to region on chromosome 2 that is associated with starch granule size in barley. Antibodies directed against encoded proteins were produced in rabbits and used in protein blotting experiments. These studies showed that both protein A and B are associated with starch granules.

Barley seeds of 152 different germplasms originating from different locations around the world have been produced under controlled conditions in a growth room. The number, diameter and volume of small and large starch granules produced were determined and found to show variation between the lines. Four lines of particular interest for their low B granule content combined with larger than normal A granules were identified.

Technical Report

Introduction and Background

The malting quality of barley grain is dependent on a complex set of traits that have been mapped to several regions on the seven barley chromosomes. Among the most important traits during malting are the activities of starch degrading enzymes, such as α-amylases, β-amylases, α-glucosidases and debranching enzymes. Other important factors affecting yield and quality of the malt extract are the properties of the starch granules.

The starch of mature barley endosperm is composed of two types of granules; large lenticular (A-type; >10 μm) and small round (B-type; < 10 μm) granules (Fig. 1). The large granules are initiated before 15 days post anthesis, whereas B granules are formed after 15 days. In mature grain, only 10-15% of the granules are of the A-type, but they represent most of the starch by weight (~90%). Large and small starch granules differ slightly in their amylose content, the overall lipid composition, and the amount of proteins attached to the surface (Morrison 1995; Meredith et al. 1981). During the mashing process in brewing, hydrolytic enzymes are more active on the large granules as compared to small granules (Borem et al. 1999), and therefore, A-type starch granules contribute more to malting quality than B-type granules. Small starch granules often cause problems during malting as they adhere easily to proteins leading to clogging of filters. Thus, there has been a desire from the malting industries to produce malt from barley lines with a lower content of small starch granules (Pierce 1991).

Fig. 1. Purified large A type and small B type granule fractions from wheat.

In an effort to identify genomic regions determining starch granule size in barley, Borem et al. (1999) analyzed a series of double haploid (DH) barley lines derived from a cross between Morex (malting) and Steptoe (feed), two cultivars that differ in granule size distribution. Marker analysis of the DH lines led to the identification of one region on chromosome 2H that was associated with A-type granule volume and proportion of A-type and B-type granules (Fig. 2). The mean maximum diameter of A-type granules correlated with a region on chromosome 5H and the shape of B granules was linked to a region on chromosome 4H. Another analysis of a wheat double haploid mapping population revealed three QTL markers affecting starch granule size (Igrejas et al., 2002). One of the QTL's was located on chromosome 4DS, which corresponds to 4H region identified in barley. The two additional QTL's were located to 7AS and 1BL. The 7AS region carries the Wx-A1 gene encoding granule bound starch synthase 1 (GBSSI), however, wheat lines carrying null GBSSI alleles show normal granule size distribution. In summary, no genetic determinant has been identified in regions associated with starch granule size in wheat or barley.

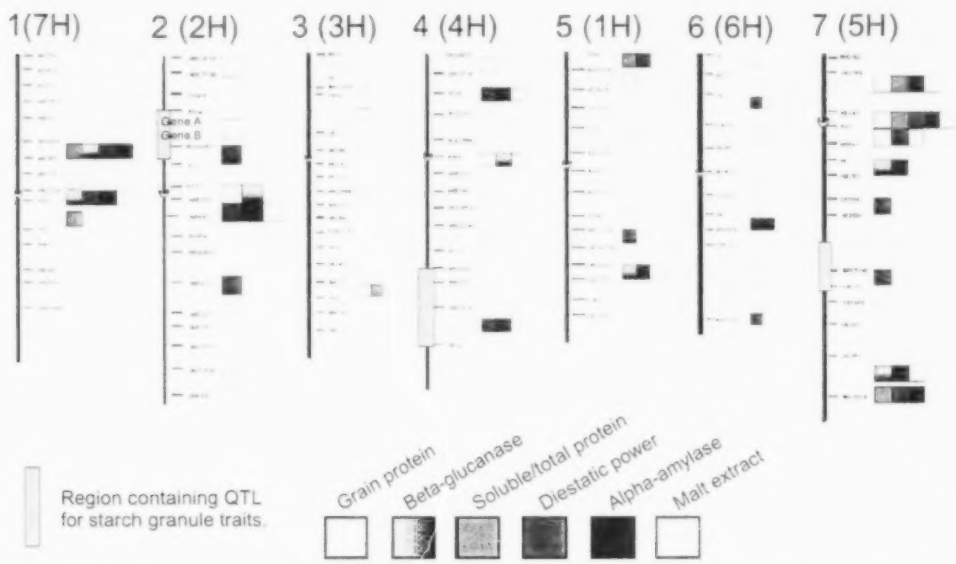

Fig. 2. Schematic illustration of genomic regions affecting malting quality in barley (http://www.css.orst.edu/barley/nabgmp/MALTQ.HTM). Regions containing QTL for starch granule traits as identified by Borem et al. (1999) are indicated by pink bars.

Production of the two size fractions of starch granules is only found in plants belonging to the Triticeae tribe, that includes wheat, barley rye and triticale. Our studies on wheat starch granules have shown that most enzymes involved in starch biosynthesis are incorporated into both small and large granules with the exception of a starch branching enzyme isoform, SBEIc, (Båga et al. 2000; Peng et al. 2000). SBEIc is a large (~150 kD) protein that, that in contrast to the well-characterized 87 kD SBEI, is preferentially incorporated into the large starch granules. SBEIc is only found in wheat, barley and rye starch granules, and thus, is associated with plants showing bimodal starch granule size distribution in the endosperm. In this project, our interest has been focused on determining the role SBEIc may play in determining the size of starch granules in plants of the Triticeae group of plants.

Results and Discussion

Plant material.
Barley accessions used in the study were provided by Plant Gene Resources of Canada, Agriculture and Agri-Food Canada and barley breeders at the Crop Development Centre, University of Saskatchewan. 152 different barley lines originating from different parts of the world have been grown under controlled conditions in the greenhouse and studied in this report. Malting, feed and non-commercially grown barley cultivars were included in the study. In addition, a wheat line with increased number of B granules and a barley mutant with reduced number of B granules were grown. Leaves, developing and mature kernels were collected from all lines.

Isolation and characterization of SBE1c homologues in barley.

Screening of barley BAC library and characterization of clones carrying SbeI sequences.
Screening of a *Hordeum vulgare* L. cv. Morex (Yu *et al.*, 2000) genomic library using a wheat *Sbe1c* probe resulted in identification of three hybridizing BAC clones. The isolated clones were found to overlap each other, and thus, their inserts were from the same region of the barley genome (Fig. 3).

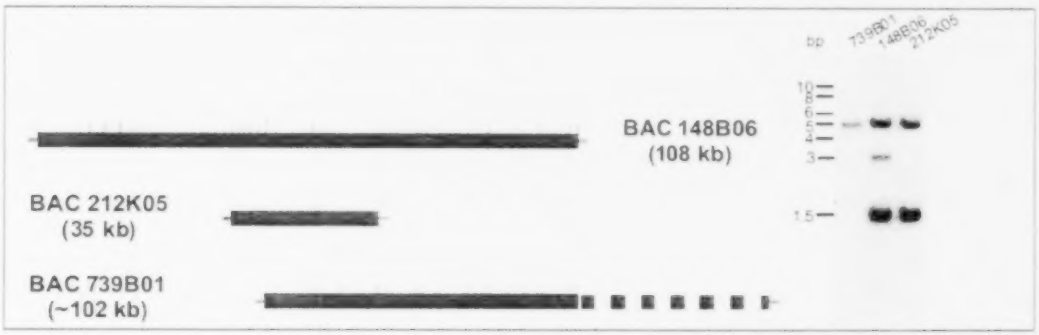

Fig. 3. Barley BAC clones carrying SbeI sequences.

To the left is shown a schematic illustration of inserts carried by the three overlapping BAC clones. DNA regions hybridizing to *Sbe1* probe are illustrated by horizontal bars. Vertical bars indicate HindIII sites.
To the rignt is shown a Southern blot analysis of EcoRI-digested DNA isolated from the BAC clones and probed with wheat *Sbe1* cDNA carrying sequences from exons 4 and 5 and first half of exon 6.

The BAC clone with the largest insert (100 kb) was subjected to shotgun cloning to generate templates for DNA sequencing reactions. Plasmid DNA was prepared using a solid phage reversible immobilization (SPRI) protocol followed by automated Ampliseq™ sequencing reactions. Purified reactions were resolved on a PE-3700 capillary sequencing device and generated sequence data was assembled into contigs using the Lasergene SeqMan (DNASTAR Inc.) software. Repetitive sequences and putative coding regions were identified using the TREP (The Triticeae Repeat Sequence Database, USDA), GENSCAN (Stanford University), the Lasergene GeneQuest (DNASTAR Inc.) and blastn, tblastx, blastp searches of nucleotide, est and protein sequence databases (NCBI). The result of the BAC sequence analysis is illustrated in Fig. 4.

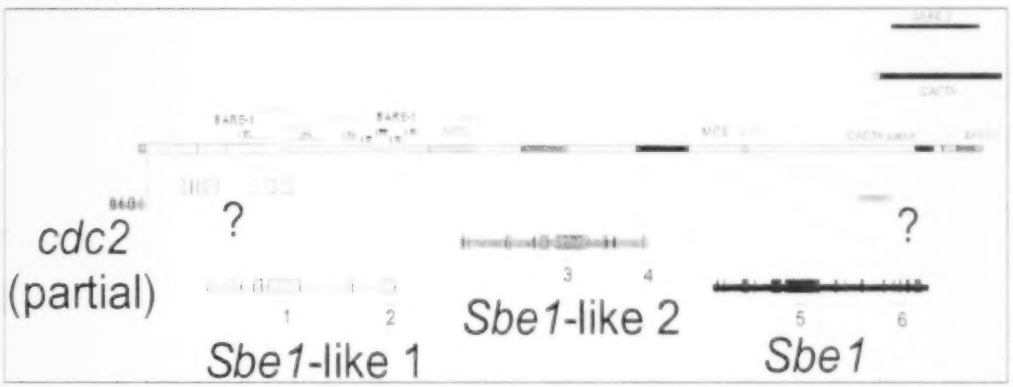

Fig. 4.
Schematic illustration of 100 kb DNA insert carried by barley BAC clone 148B06. Location of repetitive sequences and retro-transposons are indicated above the BAC DNA and coding regions of putative genes are indicated below. Locations of RT-PCR primers 1 to 6 on exon sequences are indicated.

DNA sequence analysis of BAC clone 148B06.
DNA sequence analysis of insert carried by BAC clone 148B06 revealed the presence of several repetitive sequences, including BARE-1, CACTA, BARE-2 and Sabrina-like sequences (Fig. 4). However, none of these repetitive elements is expected to be functional due to truncations, insertions and/or shifts in reading frame. Several small MITE-like and short BARE-1 LTR sequences were also found scattered on the insert. All of the small repetitive elements were located in predicted non-coding regions. From sequence alignment to barley and wheat EST and cDNA sequences deposited to the NCBI database, six putative expressed genes were identified. One of these genes encoded a complete SBEI gene, which was located in the middle of the insert. Two small *Sbe1*-like sequences were identified upstream of *Sbe1*. Sequences corresponding to the 3' end

of a *cdc2* gene and two other expressed genes of unknown function were also found on the BAC.

Identification of genetic markers for SbeIc associated with starch granule size in barley.
Analysis of the BAC 148B06 DNA sequence using the Simple Sequence Repeat Identification Tool (SSRIT; Gramene database, USDA) identified 26 simple sequence repeat (SSR) sequences. SSR sequences are generally highly variable in length among different cultivars, and thus, the repeat with its surrounding sequence can serve as marker for a specific locus on the genome. The availability of these sequences allows design of primers matching sequences surrounding the SSR loci for further use in genetic mapping. The PCR primers will be used in subsequent studies to follow inheritance of specific SBEIc, SBEI and SBEI-like genes in barley.

Alignment of SBEI and SBEI-like sequences.
Alignment of predicted exon and intron sequences of *Sbe*1 and the two small *Sbe1*-like sequences revealed large similarities. More than 78% of the amino acids encoded from the first six exons were identical between the three sequences. The largest differences between the sequences were seen at the C-terminal ends of the proteins, where the smaller SBEI-like peptides were truncated as compared to SBEI (see Fig. 5).

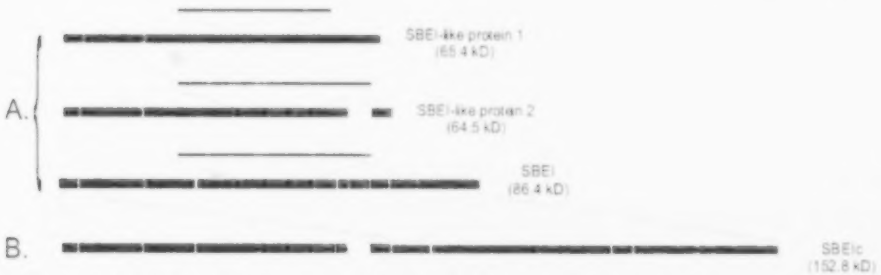

Fig. 5. Schematic alignment of SBEI and SBEI-like peptides.
Red segments represent sequences encoding peptides with high (>78%) sequence identity, whereas blue exon represents an unrelated sequence. Vertical arrows indicates predicted transit peptide cleavage sites. The four black boxes encoded from exon 6 sequences correspond to active site of starch branching enzymes. A. Alignment of peptides predicted from exons predicted for SBEI and the two SBEI-like genes. B. Structure of SBEIc. Arrows indicate sequence homology between *Sbe1c* and the *Sbe1-like 2* and *Sbe1* transcripts.

Model for SbeIc expression.
The sequence data of the BAC 148B06 clone did not indicate that a SBEIc transcript is formed from a continuous sequence of exons and introns. Rather, the *SbeIc* coding sequence is interrupted by sequences forming other *SbeI*-like transcripts. The sequence homology between *SbeIc* expressed in wheat (Båga *et al.* 2000) and the sequences predicted from the BAC clone suggest that *Sbe1*-like 2 and the *SbeI* mRNA species are involved in forming the *SbeIc* mRNA. This may involve a trans-splicing event or alternative splicing of the *SbeI*-like 2 mRNA. Further studies are required to determine which model is correct.

Expression analysis of Sbe1 and Sbe1-like genes.
To study the expression of *Sbe1* and the two small *Sbe1*-like sequences in cultivar Morex, from which the BAC clone was isolated, RT-PCR experiments using primers specific for each putative gene were done. Analysis of cDNA derived from RNA of 8 day old kernels revealed expression of *Sbe1* and both small *Sbe1*-like genes (Fig. 6). Root tissue only showed expression of *Sbe1* and leaves expressed both *Sbe1* and *Sbe1*-like gene 2. Thus, *Sbe1* and the small *Sbe1*-like transcripts are expressed when starch is accumulated in kernels. Proteins corresponding in size to peptides predicted from the Sbe1-like genes and recognized by SBEI antibodies are present in starch granules of wheat (previous data) and likely to exist in barley.

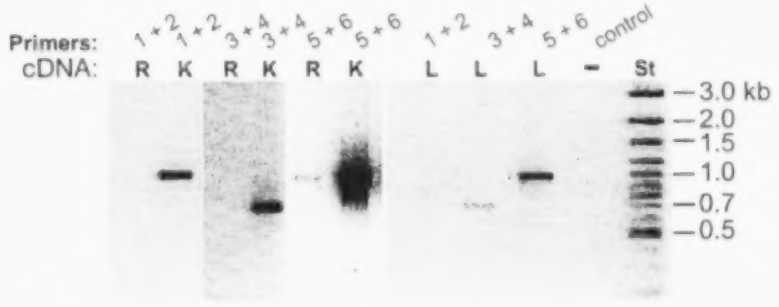

Fig. 6. Gel analysis of RT-PCR products obtained from reactions with RNA isolated from roots (R), leaves (L) and developing kernels (K) of barley cultivar Morex. Locations of the gene-specific primers used in the analysis are indicated in Fig. 4.

Identification and characterization of two new markers associated with starch granule size distribution in barley.
Analysis of gene products A and B during endosperm development.
Mapping studies by Borem et al (1999) indicated that a region on chromosome 2 (2H) plays a role in determining starch granule size (Fig. 2). We have through mapping and database searches been able to locate two genes (genes A and B) associated with glucan biosynthesis to this region of the barley genome. To study if the encoded proteins have a role in starch granule formation, we have raised antibodies against both proteins and used these for analysis of starch granules of mature barley kernels. Both gene A and gene B products were found to be present on starch granules covered with protein network (crude starch granule preparation; lanes 4, Fig. 7) and were released from the network/granules by SDS washes (lanes 1, Fig. 7). The close association of gene product A and B to the surface of starch granules further suggest that gene A and B have a role in starch granule biogenesis.

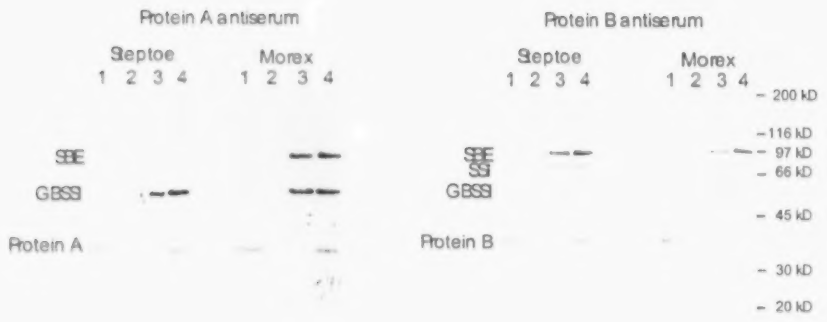

Fig. 7. Immunoblot analysis of protein A and B present in crude starch granule preparations of barley cultivars Steptoe and Morex. Proteins released from crude granules by washes containing SDS or lacking SDS are shown in lanes 1 and 2, respectively. Proteins remained attached to starch granules after +SDS wash and -SDS wash are shown in lanes 3 and 4, respectively. The antibodies raised against protein A and B show some cross-reaction with major proteins found in starch granules.

Screening of barley germplasm for variation in starch granule size distribution.

Analysis of starch granule size distribution in barley cultivars.

A low number of B granules and a large A granule volume in starch is desired for malting barley cultivars. To identify cultivars with these characteristics, we have so far screened 152 barley cultivars originating from different parts of the world. The plants were grown under controlled conditions and starch was prepared from single seeds obtained from the middle of the mature spike. More than 7,000 granules were analyzed per starch sample. The size of individual granules was determined by using an image analysis system as described by Peng et al. (2000). Compilation of results from all cultivars gave an average diameter of 5.4 um (4.7-6.3) for B granules and 19.4 (15.8-19.4) um for A granules (Fig. 8, Table 1), data in accordance with earlier studies of barley starches. The average contribution of A-granules to total volume of starch was on average 86.9% (68.0%-98.2%) for all cultivars (Fig. 9). Eight cultivars showed a low content of B granules (Fig. 10), but these numbers were often combined with low starch yields and small A granules, which indicated pre-mature termination of grain filling in the seed studied. Upon reexamination of additional seeds, five of these eight cultivars showed normal numbers of B granules. The remaining three lines showed consistent low B granule numbers and normal-size or larger A granules (Table 1) and will be used for further studies.

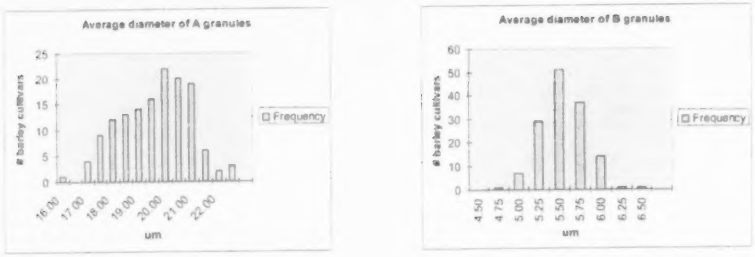

Fig. 8. Average distribution of A and B granule sizes in barley cultivars.

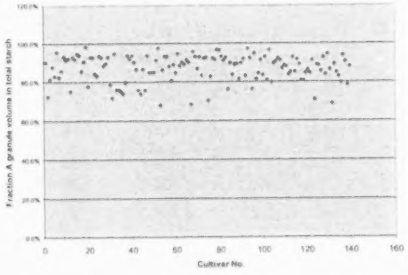

Fig. 9. Average percentage B granules in starch of total number starch granules.

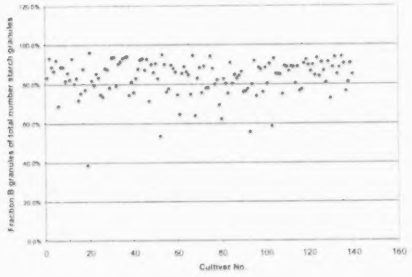

Fig. 10. Average volume of A granules in starch.

Table 1. Summary of starch granule characteristics analyzed.

Barley cultivar (um)	Volume (%)	Yield (mg)	Number (%)		Diameter
		B granules	B granules	A granules	A granules
1	9.8	68.6	5.7	20.6	95.5
2	11.1	53.4	5.8	21.2	97.5
3	7.2	69.3	5.2	21.5	96.7
All cultivars:					
Average	9.3	83.2	5.4	19.4	86.9
Max	14.4	95.3	6.3	22.2	98.2
Min	1.7	38.4	4.7	15.8	68.0

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Deviations for the proposed plan

In addition to the *Sbe1c* we have identified and characterized two additional genes associated with starch granule size distribution in barley. To study the diversity in starch granule size distribution in barley, we considered it very important to grow the plants in a green house so that we can reduce the environmental effect on starch granule development and subsequently the distribution of A- and B-type starch granules. As a result we selected 152 lines which grown in a green house. Because of judicious selection of barley germplasm, we identified three barley lines with low B-type starch granule numbers and higher A-type starch granule volume. Therefore, though we

screened fewer lines as initially proposed, we have achieved the objective of identifying barley lines with low B-type starch granules and higher A-type starch granules.

Personnel
Dr. Monica Båga – Research Scientist
Dr. Ravindra Chibbar, Molecular Biologist and Biotechnologist
Ms. Theresa Zurloff, Research Technician October 2003 to January 2004.

Equipment
None budgeted and purchased

Project Developed Materials
Antibodies to gene products A and B.
Molecular markers linked to starch granule size distribution in barley.
Barley lines with low B-type starch granules.

Project Photos
See Figures

Acknowledgement
The barley BAC clones were kindly provided by Drs Olin Anderson and Yong Gu, Genomics and Gene Discovery Unit, USDA, ARS, Albany, California, USA. Dr. Bill Crosby, PBI, is acknowledged for the use of Automation facility for DNA preparation and sequencing.

Expense statement
University of Saskatchewan Financial Services will submit the final statement.